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Environmental stress effect on the phytochemistry and antioxidant activity of a South African bulbous geophyte, *Gethyllis multifolia* L. Bolus

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ABSTRACT

Gethyllis multifolia is a South African bulbous geophyte with medicinal properties and on which very limited research has been conducted. This research investigated the effect of drought and shade, which are experienced in the natural habitat, on the antioxidant properties, as well as the isolation of natural compounds from certain plant parts. The total polyphenol, flavonol/flavone and flavanone contents, oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP) and radical cation scavenging ability (ABTS) were measured in the leaves, bulbs and roots (dry weight) of *G. multifolia* under photo- and drought stress. A significantly higher total polyphenol content was observed in the roots under the photo- and drought stresses when compared to the control. When all the plant parts were compared, the highest total polyphenol content was observed in the drought-stressed roots of *G. multifolia*. An increased antioxidant capacity was observed in the root system of *G. multifolia* where the FRAP, ORAC and ABTS were found to be significantly higher during drought stress when compared to the control. Phytochemical investigation of the leaves, bulbs and roots of *G. multifolia* revealed the presence of tannins, flavonoids, phenolics, saponins, glycosides (phenolic and terpenoid) as well as essential oils, while the test for alkaloids was negative. Further in -depth studies on the roots of *G. multifolia* led to the isolation of three known flavonoids, of which one was also isolated as its endogenously acetylated derivative. Their structures were elucidated by chemical and spectroscopic methods as 2,3-dihydro-7-hydroxy-2-phenyl-4*H*-1-benzopyran-4-one (**1**), (1-[2.4-dihydroxyphenyl]-3-phenylpropan-1-one) (**2**), 2,3-dihydro-5,7-dihydroxy-2-phenyl-4*H*-1-benzopyran-4-one or pinocembrin (**3**) and 5,7-diacetoxy-2,3-dihydro-2-phenyl-4*H*-1-benzopyran-4-one (**4**). This investigation indicated how environmental conditions can be manipulated to enhance the antioxidant properties of certain plant parts for future cultivation of this species and the isolation of the four natural compounds elucidated its medicinal potential and created a platform for future *in vivo* research.

1. Introduction

1.1. Medicinal uses and chemical composition

The genus *Gethyllis* belongs to the plant family Amaryllidaceae and is better known as "Kukumakranka" by the Khoi-San people. The genus comprises 37 currently accepted species in Southern Africa (Du Plessis and Duncan, 1989), among which many are considered to be endangered. Presently, very little is known about the chemical composition and bioactivities of this genus (Van Wyk et al., 1997). The word "Kukumakranka" is described by farmers as meaning "goed vir my krank maag" in Afrikaans, one of South Africa's eleven languages, which translates to "cure for my upset stomach" in English (Van der Walt, 2003). Watt and Breyer-Brandwijk (1962) reported that "Kukumakranka brandy", which is made from the fruit of *Gethyllis afra* and *Gethyllis ciliaris*, is believed to contain oils and esters of low molecular weight, and is an old Cape remedy that was used for colic and indigestion. According to Rood (1994) the early Cape colonialists used an alcoholic infusion of the fruit of *Gethyllis linearis* and *Gethyllis spiralis* as a remedy for digestive disturbances. In more recent times, a diluted infusion of the flower has been used for teething problems, and the skin of the fruit as a local application on boils, bruises and insect bites. Further reports by Rood (1994) indicated that the fruit was boiled by the Khoi-San and used as an aphrodisiac, while Van der Walt (2003) mentioned that *G. ciliaris* was used as a tonic for fatigue. Further pharmaceutical studies by Elgorashi and Van Staden (2004) revealed some anti-inflammatory and antibacterial activities in certain *Gethyllis* species and reported that the findings were in agreement with their uses as a traditional medicine. Previously, the following compounds: dihydroxydimethylbenzopyran-4-one, isoeugenitol, its 5-*O*-glycoside and 9Z-octadec-9-enamide, had been isolated from the roots and

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bulbs of *G. ciliaris* (Elgorashi et al., 2007). During head-space analysis of the volatiles from the fruits of *G. afra* and *G. ciliaris*, the following major compounds were characterized for *G. afra*: α-pinene, n-butyl n-butyrate, isoamyl acetate, β-pinene and 2-methylbutyl butyrate and for *G. ciliaris*: pentacosane; ethyl octanoate; ethyl isovalerate; ethyl hexanoate and ethyl benzoate. It was further reported that these compounds are responsible for the sweet/banana/piney odors of the fruit of these two species (Kamatou et al., 2008).

1.2. Botanical aspects

The interpretation of the species *Gethyllis multifolia* L. Bolus was last used in its 'Red Data Assessment' of 1996 and was classified as 'Vulnerable' (Hilton-Taylor, 1996). In the latest 'Red List of Southern African Plants' of 2009, *G. multifolia* has provisionally been subsumed under *Gethyllis campanulata*, but *G. multifolia* has not formerly been placed into synonomy with *G. campanulata* (SANBI, 2009). According to Du Plessis and Delpierre (1973) *G. multifolia* is a deciduous, winter-growing, summer-blooming and bulbous geophyte (Fig. 1A and B), 120 mm in height and indigenous to South Africa. The flowers measure 60–80 mm in diameter (Fig. 1C), colored white to cream with 12 anthers (six pairs) and the flowering period is from November to January (summer) (Goldblatt and Manning, 2000). The highly fragrant, tasty and edible fruit berries (Fig. 1D) are produced from mid-March to mid-April (autumn) at the onset of the new growing season (Van Reenen, 1975).

1.3. Biological properties

An antioxidant capacity-and -content study of plant parts of *G. multifolia* revealed higher polyphenol content and antioxidant activity in its root system when compared to the leaves and bulbs (Daniels et al., 2011). This study further revealed the highest total polyphenols and antioxidant activity in the fruits and flowers, which is comparable to blueberries, strawberries and raisins. According to Babajide et al. (2010), the brine shrimp lethality assay, which indicates toxicology levels of bioactive compounds, revealed that methanolic extracts of *G. multifolia* whole plants indicated a high potential for antimicrobial and antiviral activities. Plants possess different antioxidant properties, depending on their antioxidant molecule content, which is strongly affected by the plant's growing conditions (Lin et al., 2006). Environmental stress factors such as shade, abnormal salt levels, high temperature and drought, may result in the generation of reactive oxygen species (ROS) in plants which in turn may cause oxidative stress when in excess. In plant cells, oxidative stress reactions are associated with the production of toxic free radicals (Price et al., 1989). Plants have evolved a wide range of enzymatic and non-enzymatic mechanisms to scavenge ROS and protect their cells against oxygen toxicity (Fridovich, 1975). According to Di Carlo et al. (2001) the relationship between plant stress acclimation and human health comprises a broad array of metabolites some of which possess "desirable" pharmacological properties. Many examples can be found in nature as in the case of hyperforin, which is the active ingredient in St. John's wort (*Hypericum perforatum*) and is known for alleviating mild depression. When St. John's wort plants are subjected to heat stress it substantially increases hyperforin concentration in the shoots (Zobayed et al., 2005).

1.4. Justification for the research

According to Hilton-Taylor (1996) *G. multifolia* is threatened in its natural habitat, which stresses the need for future cultivation of this species by pharmaceutical companies, traditional healers and farmers. Should certain environmental stresses increase the antioxidant content or activity of this species, it can be incorporated in future cultivation practices to induce increased antioxidant levels in essential plant parts during production. To date no published data are available on how important biological properties, such as antioxidant activity of this *Gethyllis* species, are affected by environmental stress factors. Thus, the aim of this study was to investigate the changes in the antioxidative capacity and levels in the leaves, bulbs and roots of *G. multifolia* during controlled photo- and drought environmental stresses over one growth season. Furthermore, phytochemical screening was undertaken to isolate and characterize some natural compounds from the dried leaves,

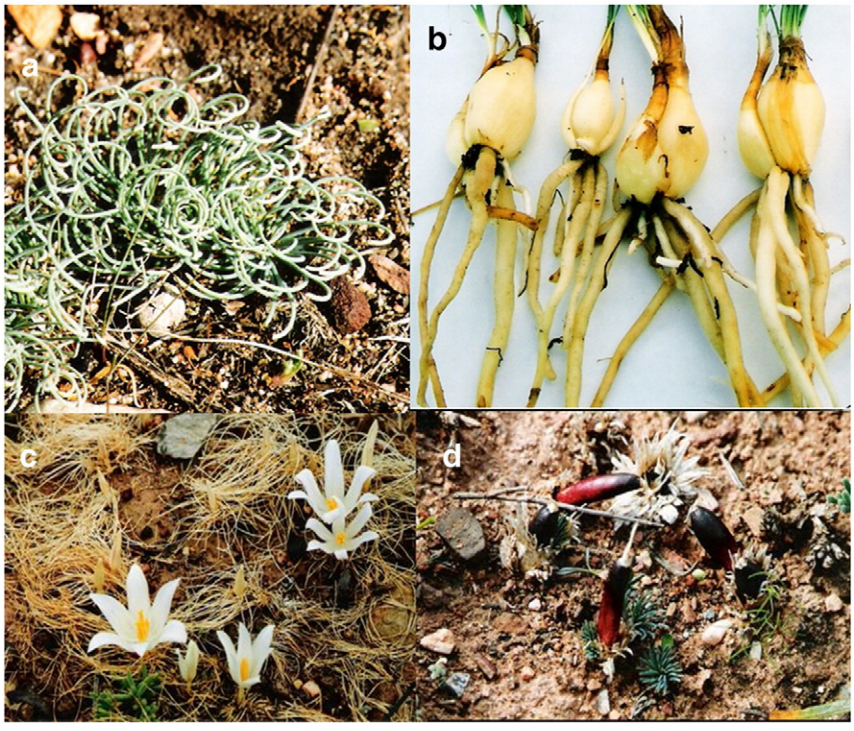

Fig. 1. The foliage emerging during March and April (a), roots and bulb (b), flowers emerging during early December (c) and the club-shaped berry (d) of *Gethyllis multifolia* protruding from the soil during March and April.

bulbs and roots of *G. multifolia* in an attempt to understand the chemistry behind the claimed medicinal values of this "Kukumakranka".

2. Materials and methods

2.1. Plant materials

G. multifolia plants were authenticated and obtained with permission from the Karoo National Biodiversity Garden (Worcester, Western Cape, South Africa) towards the end (July to mid-August) of their winter growth phase (Daniels et al., 2011). *G. multifolia* is threatened in its natural habitat, and for conservation purposes the exact location of this species is omitted. Mature dormant bulbs (size 40 mm in diameter) were selected for this investigation. The dormant bulbs were transferred into 15 cm nursery pots in sandy soil (pH 4.3–4.4) obtained from the natural habitat. The plant samples were grown for 12 months, which included one dormant phase (6 months — spring and summer) and one growth phase (6 months — autumn and winter) at the nursery of the Department of Horticultural Sciences, Cape Peninsula University of Technology (CPUT), Cape Town. Plant samples which represented the control ($n = 10$) were grown under full sunlight and irrigated by the ambient rainfall (average 85 mm/month — growth phase) of the Western Cape. The mean photosynthetic photon flux density (PPFD) on cloudless days at 1200 h was 4450 ± 155 μM m^{-2} s^{-1} (measured with a Toptronic T630 digital light meter Spraytech, Bellville, Western Cape, South Africa). Temperatures around the plant samples varied from 8–24 °C and the relative humidity from 36–100% (measured with a Majortech MT669 digital relative humidity/temperature meter, Spraytech, Bellville, Western Cape, South Africa) (Daniels et al., 2013).

Plants which represented the drought stressed samples ($n = 10$) were grown under full sunlight and covered with a 6 mm clear glass sheet, placed 300 mm above the plants. The PPFD, temperature and relative humidity environmental conditions were similar to those of the control. The drought stressed plants were irrigated at a rate of 40 mm/plant once a month with de-ionized water (Mortimer et al., 2003). Plants representing the photo-stressed samples ($n = 10$) were grown under a shade structure covered with 80% neutral black shade cloth (Alnet, Epping, Western Cape, South Africa), which has a neutral effect on light quality (Yates, 1989). During the experimental period, the mean PPFD on cloudless days at 1200 h was 570 ± 40 μM m^{-2} s^{-1} which was approximately 20% of full sunlight. The temperature around the photo-stressed plant samples was ~1–2 °C lower than that of the control, and the relative humidity 2–4% higher than that of the control. The readings of all the environmental conditions under all treatments were taken daily at the following time intervals: 0900 h, 1200 h and 1500 h.

2.2. Sample preparation

All plants were separated into leaves, bulbs and roots and dried in a fan-drying laboratory oven (Memmert, Laboratory & Scientific, Cape Town, South Africa) at 50 °C for 48 h. The bulbs had an extended drying period of five days. Individual plant parts were ground to a powder in a portable spice grinder using a 0.5 mm mesh (Krups 75 model F203, Hecho En Mexico, Mexico City, Mexico) and stored in air-tight stoppered glassware prior to analysis. Crude extracts of the leaves, bulbs and roots were prepared by stirring the various dried, powdered plant materials (0.05 g of each) in 80% (v/v) ethanol (50 mL) (EtOH) (Saarchem, South Africa) and thereafter it was centrifuged at 4000 rpm for 5 min. The supernatants were used for all analyses. The same sample preparation technique was followed for all assays and all analyses were performed in triplicate (Daniels et al., 2011).

2.3. Antioxidant content

2.3.1. Total polyphenol, flavonol/flavone and flavanone content

The total polyphenol content of the various crude extracts was determined by the Folin Ciocalteu method (Singleton et al., 1999). Using a 96-well clear microplate (visible range), 25 μL of sample was mixed with 125 μL Folin Ciocalteu reagent (Merck, South Africa), diluted 1:10 with distilled water. After 5 min., 100 μL (7.5%) aqueous sodium carbonate (Na_2CO_3) (Sigma-Aldrich, South Africa) was added to each well. The plates were incubated for 2 h at room temperature before the absorbance was read at 765 nm using a Multiskan plate reader (Thermo Electron Corporation, USA). The standard curve was prepared using 0, 20, 50, 100, 250 and 500 mg/L gallic acid in 10% EtOH and the results were expressed as mg gallic acid equivalents per g dry weight (mg GAE/g DW) (Daniels et al., 2011).

The flavonol content was determined using quercetin (0, 5, 10, 20, 40, 80 mg/L) in 95% ethanol (Sigma-Aldrich, South Africa) as standard. This assay measures both flavonols and flavones since both groups absorb ultra-violet light maximally around 360 nm. In the sample wells, 12.5 μL of the crude sample extracts was mixed with 12.5 μL 0.1% HCl (Merck, South Africa) in 95% ethanol, 225 μL 2% HCl and incubated for 30 min. at room temperature. The absorbance was read at 360 nm, at a temperature of 25 °C (Mazza et al., 1999). The results were expressed as mg quercetin equivalents per g dry weight (mg QE/g DW) (Daniels et al., 2011).

The flavanone content was determined using an adapted version of the method as described by Kosalek et al. (2004). This method was adapted with minor modifications such as reducing assay volumes for the 96-well plates. Briefly, 100 μL of sample was mixed with 200 μL [1% 2,4-dinitrophenylhydrazine (DNPH), 2% H_2SO_4 in methanol (MeOH)]. After incubation at 50 °C for 50 min., 700 μL of 10% potassium hydroxide (KOH) in 70% MeOH was added. The samples were centrifuged at 4000 rpm for 5 min. and 30 μL of the resulting supernatant mixed with 270 μL MeOH in a 96-well clear plate (visible range) and the absorbance read at 495 nm. A linear standard curve using 0, 0.2, 0.5, 1.0, 1.5, 2.0 mg/mL naringenin (Sigma-Aldrich, South Africa) in methanol was included. The results were expressed as mg naringenin equivalents per g dry weight (mg NE/g DW) (Daniels et al., 2011).

2.4. Antioxidant capacity

2.4.1. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed using the method of Benzie and Strain (1999). In a 96-well clear microplate (visible range), 10 μL of the crude sample extract was mixed with 300 μL FRAP reagent [0.3 M acetate buffer, pH 3.6 (Saarchem, South Africa), 10 mM 2, 4, 6-tripyridyl-*s*-triazine (TPTZ) in 0.1 M HCl (Sigma-Aldrich, South Africa), 20 mM iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) (Sigma-Aldrich, South Africa), 6.6 mL distilled water] and incubated for 30 min at 37 °C in the plate reader. Absorbance was measured at 593 nm. L-Ascorbic acid (Sigma-Aldrich, South Africa) was used as a standard with concentrations varying between 0 and 1 000 μM. The results were expressed as μM ascorbic acid equivalents per g dry weight (μM AAE/g DW) (Daniels et al., 2011).

2.4.2. 2,2-Azino-di-3-ethylbenzthiazoline sulfonate (ABTS) assay

The ABTS assay was performed following the method of Re et al. (1999). The stock solutions included 7 mM ABTS and 140 mM potassium-peroxodisulfate ($K_2S_2O_8$) (Merck, South Africa) solutions. The working solution was then prepared by adding 88 μL $K_2S_2O_8$ solution to 5 mL ABTS solution. The two solutions were mixed well and allowed to react for 24 h at room temperature in the dark. Trolox (6-hydrox-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard with concentrations ranging between 0 and 500 μM. The ABTS mix solution was diluted with ethanol to read the start-up absorbance (control) of approximately 2.0 (±0.1). Crude sample extracts (25 μL) were allowed to react with 300 μL ABTS in the dark at room

temperature for 30 min. before the absorbance was read at 734 nm at 25 °C in a plate reader. The results were expressed as μM Trolox equivalents per g dry weight (μM TE/g DW) (Daniels et al., 2011).

2.4.3. Oxygen radical absorbance capacity (ORAC) assay

The H-ORAC$_{FL}$ values were determined according to the methods of Prior et al. (2003) and Wu et al. (2004). A stock standard solution of Trolox (500 μM) was diluted in phosphate buffer (75 mM, pH 7.4) to provide calibration standards ranging from 5 to 25 μM. The Fluoroskan ascent plate reader (Thermo Fisher Scientific, Waltham, USA) equipped with an incubator was set at 37 °C. Fluorescence filters with an excitation wavelength of 485 nm and emission wavelength of 538 nm were used. A fluorescein stock solution was prepared in a phosphate buffer and further diluted to provide a final concentration of 14 μM per well. The peroxyl generator, 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH) (25 mg/mL in a phosphate buffer), was added with a multichannel pipette to give a final AAPH concentration of 4.8 mM in each well. The fluorescence from each well, containing 12 μL diluted hydrophilic extract, was read every 5 min. for 2 h. The final ORAC$_{FL}$ values were calculated using the regression equation $y = ax^2 + bx + c$ between the Trolox concentration (μM) and the area under the curve. The results were expressed as μM Trolox equivalents per g dry weight (μM TE/g DW) (Daniels et al., 2011).

2.5. Statistical analysis

The statistical significance between antioxidant content and activity values of the various crude plant extracts were determined by an analysis of variance (ANOVA) where $P < 0.05$ was considered to be statistically significant. The computer software employed for the statistical analysis was Medcalc version 9.4.2.0 (Medcalc, Belgium). The computer program, Microsoft Office Excel 2006, version 12 (Microsoft Corporation, USA) was employed to determine the correlation between antioxidant contents and activity.

2.6. Phytochemistry

2.6.1. General methods

All laboratory grade solvents (Merck, South Africa) were distilled prior to use and all spectroscopic grade solvents were used as such. Cleaning up of crude isolates was performed using Sephadex LH-20 (Sigma-Aldrich, South Africa). Preparative thin layer chromatography (TLC) (Sigma-Aldrich) was performed using Merck Silica gel 60 PF_{254} on glass plates (20 cm × 20 cm) with a thickness of 0.5 mm. Analytical TLC was conducted on normal-phase Merck Silica gel 60 PF_{254} precoated aluminium plates. Separated compounds on TLC were visualized under ultra-violet (UV) light at (254 and 360 nm) and spraying of the plates where required, was carried out using 2% vanillin in H_2SO_4, followed by heating at 120 °C for 3–4 min. All extracts were concentrated on a rotary evaporator (Buchi Rotavator R-114, Germany) at 45 °C. Column chromatography was performed using Merck Silica gel 60 H (0.040 − 0.063 mm particle size, Merck). Melting points (m.p.) were determined on a Fisher-John's melting point apparatus (Fisher-Scientific). Ultra-violet spectra (UV) of some of the isolated compounds were obtained with a Unicam UV4-100 UV/Vis Recording Spectrophotometer. Infra-red (IR) spectra were recorded on a Perkin Elmer Universal ATR (Precisely) Spectrum 100 series FT-IR spectrometer. Mass spectrometry (MS) was performed on a Waters Synapt G2API Q-TOF Ultima LC–MS-ESI instrument in the positive mode, while nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova 600 MHz NMR spectrometer in MeOH-d_4, using the solvent signals as internal reference.

2.6.2. Extraction of the constituents

The dried, powdered parts of the plants, namely the leaves, bulbs and roots were separately extracted and the dry weights (DW) for excised plant parts were as follows: leaves (7.0 g), bulbs (10.0 g) and roots (9.9 g). Extraction was carried out sequentially using hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH). Extraction was done under the ambient light conditions of the laboratory facility. By means of occasional stirring using a mechanical stirrer, each portion of plant material was macerated twice in 250 ml of each solvent at room temperature (25 °C) for 24 h, and the extracts were evaporated on a rotary evaporator at 45 °C. Each extract was screened for the presence of tannins, flavonoids, phenolics, saponins, glycosides (phenolic and terpenoid), alkaloids, steroids, essential oils and terpenes according to the method of Wagner and Bladt (2001). Since the chromatographic profiles of the leaves and bulbs were similar, while higher recoveries were indicated in the roots (data not shown), a more in-depth investigation of the natural product content was thus conducted on the roots of *G. multifolia*. Furthermore, the chromatographic profiles for the abovementioned plant parts were also found to be similar for the control and photo- and drought stress treatments.

2.6.3. Column chromatography

The height of the column was 750 mm with an internal column diameter of 25 mm. The flow rate of the eluent was measured at 2 ml/min and the volumes collected were 10 ml in Pyrex test tubes. The ethyl acetate extract (5.0 g) of the root of *G. multifolia* was adsorbed on silica gel 60 and chromatographed using the solvent mixtures: 100 ml of toluene and then 50 ml each of the following mixtures, toluene–EtOAc (90:10), (80:20), (60:40), (20:80) and (10:90). This was followed by EtOAc (50 ml) and 50 ml each of the following mixtures, EtOAc–MeOH (90:10), (80:20), (60:40), (20:80), (10:90). Finally the column was washed with 70 ml of MeOH. Fractions (10 ml) collected were analyzed by TLC using toluene: EtOAc:MeOH (5: 4: 3). Fractions showing the same TLC profile were pooled and concentrated *in vacuo*. Three distinct major fractions were selected and coded A–C. Fraction B (1.32 g) was rechromatographed using the same solvent mixtures, column diameter and flow rate as described above. Out of the four fractions collected (on the basis of TLC profiles), fraction B (iii) was chromatographed on Sephadex LH 20 using toluene–MeOH (7:3), to yield a brownish-yellow powder, which upon further purification by preparative TLC using toluene:EtOAc:MeOH (5:4:3), afforded compound **1** (GT1) (22 mg). Fraction C (0.5 g) was rechromatographed using the same solvent mixtures as shown above. Successive chromatography, followed by preparative TLC of the fraction obtained using toluene–MeOH (7:3), afforded compound **2** (GT2) (12 mg) as an off-white powder. Although this isolate (compound **2**) appeared as a single spot on TLC, MS and NMR, it was subsequently revealed to be a mixture of two compounds.

The methanol extract of the root (5.0 g) of *G. multifolia* was chromatographed using the same solvent mixtures as for the EtOAc extract, except that the final volume of MeOH was 80 ml. Sixty-five (10 ml) fractions were analyzed by TLC and those showing the same profile were pooled. Five fractions coded D − H, were obtained. One of the fractions F (0.9 g) was rechromatographed using the same solvent mixtures. Repeated preparative TLC afforded compound **3** (GT3) (11.1 mg) as a dull, yellow powder. Compound **3** (as indicated here) becomes compound **4** in the 'Results and discussion' section because the isolated compound **2** is a mixture of two different compounds, and was discussed as such.

3. Results and discussion

3.1. Antioxidant-capacity and -content

3.1.1. Total polyphenol, flavonol/flavone and flavanone content

In general, the antioxidant-capacity and -content fluctuated in the leaves and roots of *G. multifolia* compared to low and stable activity in the bulb, when plant parts were subjected to both environmental stresses. When compared to the control, it was evident in this investigation that the total polyphenol and flavonol/flavone content increased in

the roots of *G. multifolia* when the plants were subjected to the drought stress (Fig. 2A and B). In comparison to a previous investigation of natural populations of the same species which were not subjected to any form of environmental stress factors, the root system in this investigation under drought stress indicated the highest polyphenol and flavonol content for both studies (Daniels et al., 2011). Though not significant, higher flavonol/flavone levels were also recorded in the leaves when subjected to the drought stress treatment. Similarly, it was reported that in *Arbutus unedo* plants, severe drought stress resulted in significantly higher ascorbate levels in plant parts (Munne-Bosch and Penuelas, 2004). Furthermore, a study by Herbinger et al. (2002) reported that under drought stress, α-tocopherol and glutathione concentrations increased in certain wheat cultivars. In contrast to the above reports, a study by Kirakosyan et al. (2003) reported that drought stress effected a reduction in the flavonoid levels of *Crataegus laevigata* and *Crataegus monogyna* plants. The increase in the total polyphenols and flavonol/ flavone content of *G. multifolia* when subjected to drought stress, could suggest that this species produces higher levels of total polyphenol antioxidants to possibly serve as a protective measure or adaptive strategy to cope with this specific environmental stress.

Furthermore, *G. multifolia* significantly decreased its flavanone content in all plant parts when the plants were subjected to the photo-stress (deep shade) treatment (Fig. 2C). Evidence of similar plant responses to light was mentioned in a study by Tattini et al. (2004) where high light intensities effected an increase in the flavonoid concentrations in the leaves of *Ligustrum vulgare*. Furthermore, Heuberger et al. (2004) reported that under chronic UV-exposure conditions the ascorbate levels in certain plant species increased, which reflected the stress acclimation process. In support of the above observations, Dixon and Paiva (1995) reported that plants which are subjected to full-sun conditions have been shown to contain higher levels of polyphenolic compounds than shade plants. These findings are line with a previous comparative investigation of the natural habitat of two species of *Gethyllis*, which suggested that *G. villosa* adapts better than *G. multifolia* when exposed to seasonal drought periods (Daniels, 2007).

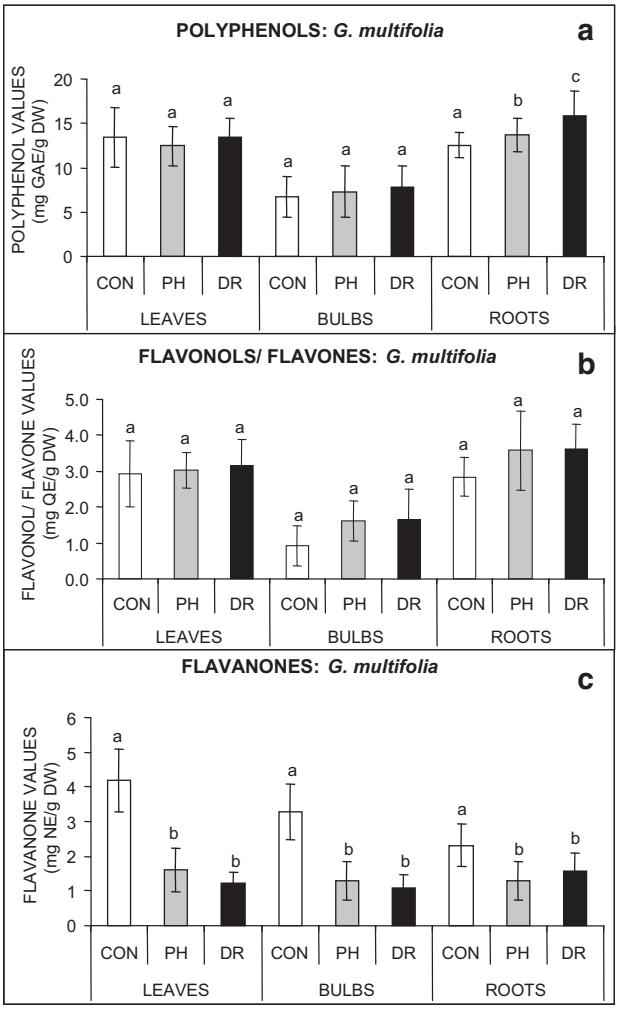

Fig. 2. The total polyphenol (mg GAE/g dry weight), flavonol/ flavone (mg QE/g dry weight) and flavanone (mg NE/g dry weight) content of the leaves, bulbs and roots of *Gethyllis multifolia* plants under different environmental stresses. Values represent the means ± SD for the leaves, bulbs and roots (n = 10). Environmental stresses are compared to the control of each plant part. Means with different letters for the same plant part are significantly ($P < 0.05$) different. CON: control; PH: photo-stress; DR: drought stress; GAE: gallic acid equivalents; QE: quercetin equivalents and NE: naringenin equivalents.

3.1.2. Antioxidant capacity

The FRAP was found to be significantly higher in the roots under the photo-stress and drought stress treatments for *G. multifolia* (Fig. 3A). This response was also evident when this investigation was compared to a previous study on natural populations of *Gethyllis multifolia* and *Gethyllis villosa* which were not subjected to environmental stress factors (Daniels et al., 2011). Similar higher reduction ability responses were reported when sweet potato leaves were subjected to drought stress (Lin et al., 2006). The ORAC values increased significantly in the underground organs (bulbs and roots) of *G. multifolia* after exposure to the shade- and drought stress treatments, but effected a significant decrease in the aerial parts (Fig. 3B). Conversely, increased ORAC values were recorded in the leaves of Sushu 18 and Simon 1 when these two sweet potato cultivars were subjected to the drought stress treatment (Lin et al., 2006). This investigation, however, revealed that under the drought stress treatment the FRAP, ORAC and ABTS radical cation scavenging ability is significantly increased in the root system of *G. multifolia*, which could form part of the acclimation process or adaptive strategy to this environmental stress factor (Fig. 3A, B and C) These findings are further supported by a previous comparative study on natural populations (no subjection to environmental stress factors) where no increases in the ABTS and ORAC levels in the root system were evident (Daniels et al., 2011).

3.2. Phytochemistry

According to Fennell and Van Staden (2001), the majority of compounds found in the Amaryllidaceae family are usually alkaloids. Further studies reported that alkaloids were not detected in either the dichloromethane or 90% methanolic extracts of *G. ciliaris*, using Dragendorff's reagent; only tannins, flavonoids, phenolics, saponins, anthraquinones, glycosides and essential oils tested positively in all the extracts. A study conducted by Babajide et al. (2010) also revealed the presence of the same phytochemical compounds, and the absence of alkaloids from the methanol and water extracts of *G. multifolia* and *G. villosa* whole plants. The latter study also revealed the absence of saponins in the water extracts for both species, an observation that was further confirmed in all the plant parts in this study (data not shown). Earlier reports by Viladomat et al. (1997) had revealed that the bulbs of *Gethyllis* species also contained flavonols, organic acids, carbohydrates and soluble nitrogen compounds.

The preliminary phytochemical screening (MeOH extract) results in this investigation indicated the presence of tannins, flavonoids, phenolics, saponins, glycosides as well as essential oils, while the tests were negative for alkaloids in all the plant parts tested (data pooled). The following compounds were isolated from the roots by means of column chromatography:

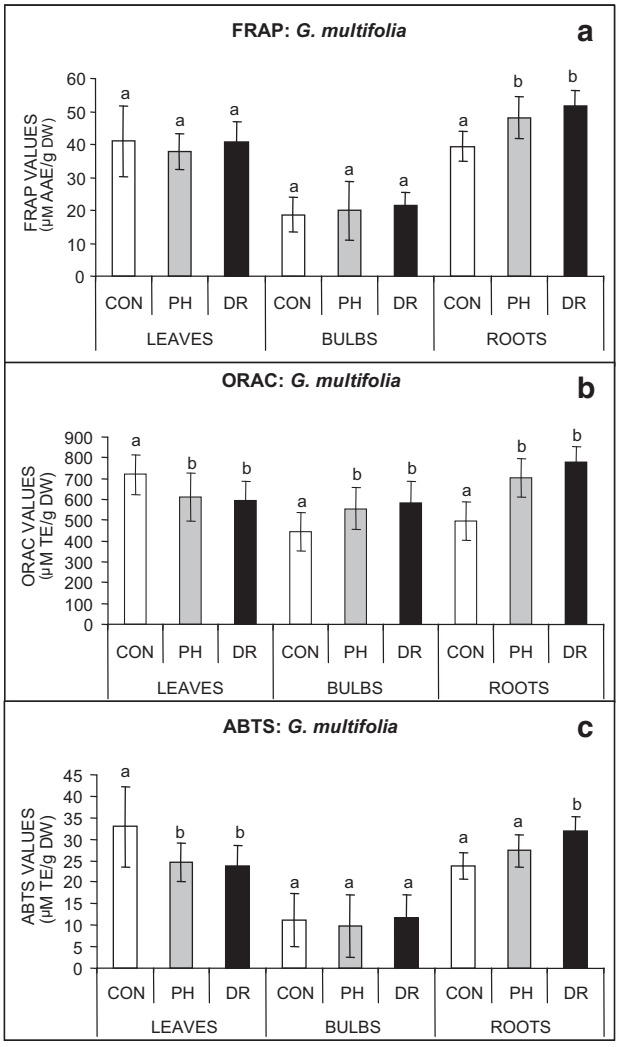

Fig. 3. The ferric reducing antioxidant power (FRAP) (μM AAE/g dry weight), oxygen radical absorbance capacity (ORAC) (μM TE/g dry weight) and ABTS radical cation scavenging ability (ABTS) (μM TE/g dry weight) of the leaves, bulbs and roots of *Gethyllis multifolia* plants under different environmental stresses. Values represent the means ± SD for the leaves, bulbs and roots (n = 10). Environmental stresses are compared to the control of each plant part. Means with different letters for the same plant part are significantly ($P < 0.05$) different. CON: control; PH: photo-stress; DR: drought stress; AAE: ascorbic acid equivalents and TE: Trolox equivalents.

Compound **1** (2,3-dihydro-7-hydroxy-2-phenyl-4*H*-1-benzopyran-4-one)
A brownish-yellow powder (22 mg) with m.p. 203–205 °C.
UV λ_{max} nm (log ε): 261, 365; 262, 366; 280, 443; 268, 412.
IR (ν cm^{-1}) pronounced peaks: 3400, 2931, 1656, 1615 and 1556.
ESI-MS: *m/z* 241.08 ($[C_{15}H_{12}O_3 + H]^+$); ^{1}H NMR δ 7.73 (1H, d, J8.2 Hz, H-5), 7.49 (2H, m, H-2′,6′), 7.40 (2H, m, H-3′,5′), 7.35 (1H, m, H-4′), 6.51 (1H, dd, J8.2 Hz and 2.0 Hz, H-6), 6.39 (1H, d, J2.0 Hz, H-8), 5.48 (1H, dd, J12.8 and 3.0 Hz, H-2), 3.02 (1H, dd, J17.0 and 12.8 Hz, H-3a), 2.75 (1H, dd, J17.0 and 3.2 Hz, H-3a); ^{13}C NMR δ 193.0 (C-4), 166.8 (C-7), 165.4 (C-8a), 140.7 (C-4′), 129,9 (C-5), 129.7 (C-1′), 129.5 (C-3′,5′), 127.3 (C-2′,6′), 115.1 (C-4a), 111.9 (C-6), 103.9 (C-8), 81.0 (C-2), 49.4 (C-3).
The molecular formula of compound **1** (Fig. 4) was determined as $C_{15}H_{12}O_3$ by ESI-MS, on the basis of the pseudomolecular ion peak at *m/z* 241.08 $[M + H]^+$. The flavanone characteristics were evident from the presence of an ABX spin system due to the protons H-3e, H-3a and H-2, along with the typical coupling constants. Further evidence for the structural assignment came from both 1-D and 2-D NMR measurements (COSY, HSQC, HMBC and NOESY). H-5 appeared as the most deshielded proton at δ 7.73 due to its occupancy of a β position in an α-β unsaturated carbonyl system. This observation, along with the complete absence of the familiar chelated OH-5, characteristic of most naturally occurring flavonoids, was indicative of the fact that this was one of those unusual flavonoids. This flavonoid has recently been reported as isolated from *Zuccagnia punctata* (Zampini et al., 2012), *Spatholobus suberectus* (Shim, 2011) and *Dalbergia cochinchinensis* (Shirota et al., 2003), among many other sources. Its synthesis has also been reported for the purpose of crystallographic and conformational studies (Kendi et al., 1995).

Compound **2** (1-[2.4-dihydroxyphenyl]-3-phenylpropan-1-one) and 3 (2,3-dihydro-5,7-dihydroxy-2-phenyl-4*H*-1-benzopyran-4-one or pinocembrin)
An off-white powder (12 mg) with m.p. 225–227 °C.
IR (ν cm^{-1}) pronounced peaks: 3385, 2828, and 1690.

2; ESI-MS:*m/z* 243.10 ($[C_{15}H_{14}O_3 + H]^+$); ^{1}H NMR δ 12.70 (1H, s, 2′-OH), 7.67 (1H, d, J9.6 Hz, H-6′), 7.23 (5H, m, H-Aryl), 7.14 (1H, d, J2.1 Hz, H-3′), 6.32 (1H, dd, J9.6 and 2.1 Hz, H-5′), 3.20 (2H, t, J7.1 Hz, H-2), 2.98 (2H, t, J7.1 Hz, H-3); ^{13}C NMR δ 205.2 (C-1), 133.7 (C-6′), 129.4 (C-Aryl), 127.1 (C-3′), 109.1 (C-5′), 40.5 (C-2), 31.6 (C-3).

3; ESI-MS:*m/z* 257.08 ($[C_{15}H_{12}O_4 + H]^+$); ^{1}H NMR δ 12.05 (1H, s, 5-OH), 7.47 (2H, dd, J7.5 and 1.5 Hz, H-2′,6′), 7.39 (2H, m, H-3′,5′), 7.34 (1H, m, H-4′), 6.24 (1H, d, J2.2 Hz, H-8), 5.91 (1H, d, J2.2 Hz, H-6), 5.41 (1H, dd, J12.8 and 3.2 Hz, H-2), 3.05 (1H, dd, J12.8 and 17.2 Hz, H-3a), 2.74 (1H, dd, J17.2 and 3.2 Hz, H-3e); ^{13}C NMR δ 197.3 (C-4), 129.7 (C-3′,5′), 129.6 (C-4′), 127.3 (C-2′,6′), 103.7 (C-8), 97.2 (C-6), 80.4 (C-2), 44.2 (C-3).

Compound **4** (5,7-diacetoxy-2,3-dihydro-2-phenyl-4*H*-1-benzopyran-4-one)
A dull, yellow powder (11.1 mg) with m.p. 215–217 °C.
IR (ν cm^{-1}) pronounced peaks: 3500–3400, 1652, 1613, 1568, 1556.

ESI-MS:*m/z* 341.10 ($[C_{19}H_{16}O_6 + H]^+$) and m/z 363.08 ($[C_{19}H_{16}O_6 + Na]^+$); ^{1}H NMR δ 7.45 (2H, dd, J7.3 and 1.5 Hz, H-2′,6′), 7.41 (2H, m, H-3′,5′), 7.36 (1H, m, H-4′), 6.81 (1H, d, J1.7 Hz, H-6), 6.60 (1H, d, J1.7 Hz, H-8), 5.56 (1H, dd, J13.2 and 2.8 Hz, H-2), 3.10 (1H, dd, J16.7 and 13.2 Hz, H-3a), 2.77 (1H, dd, J16.7 and 2.8 Hz), 2.31 (3H, s, 5-CH_3CO), 2.27 (3H, s, 7-CH_3CO), ^{13}C NMR δ 191.1 (C-4), 170.9 and 169.7 (2X C = O, CH_3CO), 129.8 (C-3′,4′,5′), 127.4 (C-2′,6′), 111.7 (C-8), 110.3 (C-6), 81.0 (C-2), 45.9 (C-3).

The molecular formula of compound **3** (Fig. 4) was determined as $C_{15}H_{12}O_4$ by ESI-MS, on the basis of the pseudomolecular ion peak at *m/z* 257.08 $[M + H]^+$). NMR spectroscopy revealed features of a flavanone structure, as well as a chelated OH. Comparison with literature data (Wollenweber, 1982), suggested the structure to be that of a well-known flavanone, pinocembrin. Pinocembrin has been shown to be a flavonoid constituent in several plants, such as litchi (Wang et al., 2011) and has reportedly displayed antibacterial and antifungal activity *in vitro* (Bremner and Meyer, 1998). Furthermore, Ahn et al. (2009) reported that pinocembrin exhibited very low antioxidant activity but possessed a considerable degree of antiangiogenic activities. Although the phenolic group is considered to be the most fundamental structural

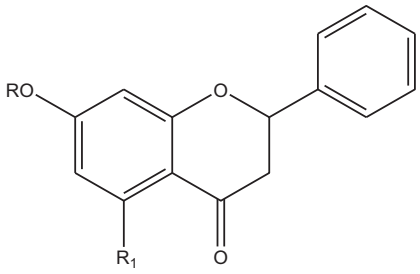

Fig. 4. Compound **1**; R, R_1 = H. (2,3-Dihydro-7-hydroxy-2-phenyl-4*H*-1-benzopyran-4-one). Compound **3**; R = H, R_1 = OH. (2,3-Dihydro-5,7-dihydroxy-2-phenyl-4*H*-1-benzopyran-4-one or pinocembrin). Compound **4**; R = Ac, R_1 = OAc. (5,7-Diacetoxy-2,3-dihydro-2-phenyl-4*H*-1-benzopyran-4-one).

feature essential for antioxidant activity, the multiple presence of this functionality, as well as their specific locations on the flavonoid skeleton relative to other functional groups, has been shown to be critical for the enhancement of antioxidant activity in selected flavonoids. Thus, the presence of one or more of the following features in a flavonoid is known to contribute to enhanced antioxidant activity: **(1)** the pyrogallol group; **(2)** the catechol group; **(3)** the 2,3-double bond in conjugation with a 4-oxo group and a 3-hydroxyl group; and **(4)** additional resonance-effective substituents (Janeiro and Oliveira-Brett, 2004).

Compound **4** (Fig. 4) had a molecular formula of $C_{19}H_{16}O_6$ as determined by ESI-MS, {pseudomolecular ion peaks at *m/z* 341.10 ($[M + H]^+$) and 363.08 ($[M + Na]^+$)}. The NMR spectroscopic features of **4** were very similar to those of **3**, except that **4** carried two acetyl groups at O positions 5 and 7 (δ2.27 and 2.31), and of course showed no evidence for a chelated OH. The presence of the acetyl groups was also evident from the significant downfield shift observed for H-6 and H-8, as well as the appearance of two carbonyl ^{13}C signals at δ 170.9 and 169.7, in addition to δ 191.1 for C-4. Deacetylation of **4** with sodium methoxide in methanol yielded a mixture of **3** and the partially deacetylated product (5-acetoxy-2,3-dihydro-7-hydroxy-2-phenyl-4*H*-1-benzopyran-4-one).

The occurrence of the latter compound as a by-product may be attributed to some degree of hindrance from the C-4 carbonyl group to the approach of the methoxide anion. Furthermore, acetylation of pinocembrin gave a product whose spectral data was identical to that of **4**. The occurrence of pinocembrin as its diacetylated form in nature has, to our knowledge, not been reported previously. However, based on the existence of a broad spectrum of acetyl transferases in plants (Hampel et al, 2006), it is possible that similar enzymes may be responsible for the production of this diacetate. On the other hand, that this compound may be an artifact cannot be ruled out, especially given that ethyl acetate was a prominent solvent constitutent of the eluent during column chromatography. The acetyl group has been shown to migrate between hydroxyl groups in the presence of a suitable catalyst, such as certain brands of silica gel.

Compound **2** (Fig. 5) displayed a molecular formula of $C_{15}H_{14}O_3$ as determined by ESI-MS, pseudomolecular ion peak at *m/z* 243.10 ($[M + H]^+$). It also displayed the characteristic AB spin system usually observed for dihydrochalcones. This dihydrochalcone, which was also shown to possess a chelated OH group (δ 12.70) was assigned the proposed structure on the basis of 1-D and 2-D NMR spectral analysis. This compound has previously been reported both as a synthetic (Nakamura et al., 2003) as well as a natural (Vries et al., 2005) product.

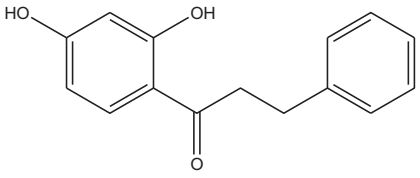

Fig. 5. Compound **2**. (1-[2,4-Dihydroxyphenyl]-3-phenylpropan-1-one).

4. Conclusion and future research

It can be concluded that the antioxidant activities in the mentioned plant parts under drought stress may be a protective and acclimation mechanism against drought stress, which is found to be very significant in the root system of this species of *Gethyllis*. The responses of plants in this investigation have also given a good indication as to how the different plant organs respond to different environmental stresses by increasing and decreasing their secondary metabolites or changing their organ morphology and physiological processes as possible protective mechanisms (Daniels et al., 2013). Since the flavonoids reported in this paper lack these key features, while they will contribute to the total polyphenolic content, they may not be considered to be the key contributing compounds towards the antioxidant activities which have been reported for the roots in this investigation. Results from this study could have a significant impact on how traditional healers, pharmaceutical companies and farmers choose conducive environmental conditions for the cultivation of this *Gethyllis* species in order to ensure enhanced polyphenolic content and antioxidant activities in the relevant plant "parts" that are traditionally used in medicinal practices. Future research is needed on the effect of drought on the antioxidant activities of the flowers and fruit, and how irrigation strategies can be effectively manipulated to increase biomass yield of this species but still simulate the effect of drought. Further research is also warranted on the flowers and fruit of *G. multifolia* to isolate more natural compounds and to further elucidate other biological properties of this endemic plant species, but also to confirm the antioxidant activity and other medicinal benefits *in vivo*.

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